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Award Number: DAMD17-99-2-9027

TITLE: Drug Development and Conservation of Biodiversity in West and Central Africa

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REPORT DATE: May 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual (1 May 2002 - 30 Apr 2003)

4. TITLE AND SUBTITLE
Drug Development and Conservation of Biodiversity in West and Central Africa

5. FUNDING NUMBERS
DAMD17-99-2-9027

6. AUTHOR(S)
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Pace University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This report concerns activities of Associated Project #3 (AP#3) of the International Cooperative Biodiversity Group Program (ICBG) directed by the Walter Reed Army Institute for Research (WRAIR). This project examines medicinal plants of Central and West Africa as anti-typanosomal and anti-trichomonal agents. In the reporting period 85 plant extracts were received from WRAIR and 34 were received from the University of Dschang (Cameroon, AP#2). Extracts were screened in vitro vs. blood stream forms of African trypanosomes (Trypanosoma brucei, Trypanosoma rhodesiense) and pathogenic trichomonads (*Trichomonas vaginalis, Tritrichomonas foetus*).

In the trypanosome growth screen, 58 WRAIR extracts and 8 AP#2 extracts were tested. Eight WRAIR extracts were tested vs metronidazole-resistant and -senstive strains of *T.vaginalis* and another 31 were studied in a *T. foetus* screen. Of 58 WRAIR extracts tested 24 had IC_{50} values <20 µg/ml in the trypanosome screen. For trichomonads 2 of 8 WRAIR extracts had MIC values <0.6mg/ml for metronidazole-resistant *T. vaginalis*; 4 0f 31 extracts were similarly active for *T. foetus*. For trypanosomes, plant genera yielding active extracts included: *Melian, Holarrhena, Jatropha* and *Boerrhavia*. For trichomonads, active extracts were from: *Aspilia, Combretum, Enantia, Hoslundia, Mormadica, Phyllanthus* and *Cleistophotis*.

14. SUBJECT TERMS
No subject terms provided.

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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I. Introduction

This project concerns drug discovery and development through ethnobotanical leads to agents in growth screens against African trypanosomes and pathogenic trichomonads. These diseases are of significant concern in continental Africa and elsewhere in developing countries, particularly those in which AIDS is prevalent. In some cases disease is cosmopolitan (*Trichomonas vaginalis*), in some areas, new human epidemics have begun (African human trypanosomiasis, e.g, *Trypanosoma rhodesiense*) and in some instances diseases pose significant threats to livestock and their breeding (*Trypanosoma brucei* and *Tritrichomonas foetus*). This ICBG program is under the direction of WRAIR and concerns plant extracts from Central and West Africa. These are supplied by ethnobotanists and chemists at WRAIR and through the Department of Chemistry, University of Dschang (Cameroon, AP#2).

Human African trypanosomiasis is endemic over 10 million square kilometers of sub-Saharan African, affecting human as well as all domesticated livestock (WHO, 1995). Recently, the number of new human cases of sleeping sickness has escalated from ~ 25,000/year to 500,000/year and an incidence of veterinary sickness of 300,000 cases (WHO 2001, van Nieuwenhove et al. 2001). These estimates are most likely low, based on civil unrest and lack of local medical surveillance (F. Kuzoe, pers. Commun.). The major drugs for human disease, pentamidine and melarsorpol (Absorbal®) have been in use > 50 years. These agents, particularly melarsoprol, have associated CNS and other toxicity, and their continued used has led to increased incidence of resistance (van Nieuwenhove, 1992; Kuzoe 1993; Wery 1994). Melarsoprol remains the only drug in common use for late stage CNS disease (Burri and Keiger 2001; Legros et al., 1999).

Trichomonas vaginalis is a sexually transmitted human pathogen of the urogenital tract. It affects the vaginal epithelium causing severe irritation. Trichomonisais one of the most prevalent STDs in the western world (Lossik, 1989; Hammill 1989). Recent evidence suggests a high incidence rate between cervical cancer and trichomoniasis. In the United States alone, there are ~ 3 million reported cases (Hook 1999). Studies in developing countries and cosmopolitan areas of large cities indicate the concurrent presence of *T. vaginalis* infection and exposure to HIV-1 in semen is an important additional risk for contraction of AIDS (Laga et al., 1993; Sorvillo and Kerndt 1998; Hook 1999; Jackson et. al., 1997). The single major treatment for human trichomoniasis is a 5-nitroimidazole, metronidazole (Fagyl®), which has been in continuous extensive use since 1955 in the United States and Europe; drug-resistant strains are becoming more prevalent (Meingassner and Thurner 1979; Voolman and Boreham 1993; Wong et. al., 1990). It is potentially mutagenic, based on ability to form free radicals and is not given to pregnant women (Lossick 1989). At present there is no alternative therapy for metronidazole-refractory disease or for pregnant women.

Trichomonas foetus is the agent of bovine trichomoniasis, causing reproductive failure. Parasites are spread by infected bulls and cause abortion of the fetus. In some cases, the cow is permanently sterilized. There is no satisfactory treatment of infected bulls since metronidazole kills the rumen flora. Unless the bull is valuable, it is usually destroyed (Levine 1985).

During the reporting period, a total of 85 plant extracts were received from WRAIR and 34 from the group at the University of Dschang. Many of these agents have been tested *in vitro* in bloodstream forms of African trypanosomes (*Trypanosoma brucei* Lab 110 EATRO, veterinary parasite; *Trypanosoma rhodesiense* KETRI 243 and *T. rhodesiense* 243AS10-3, human parasites) vs. metronidazole-sensitive and –refractory *Trichomonas vaginalis* (C1-NIH/ATCC30001 and ATCC50143/CDC-085, respectively). Sixteen plant extracts having high *in vitro* activity were also tested in a *T. brucei* mouse model infection.

II. Body

1. Methods

a) African trypanosomes. *In vitro* screens with bloodstream form trypanosomes are set up in 24 well plates using duplicate wells of four extract concentrations each (in HMI medium: Hirumi & Hirumi, 1989) plus full-growth controls, as detailed in Bacchi et al. (1997). Initial wide concentration curves were followed by narrow-ranging curves to determine IC_{50} values. Strains of trypanosomes used were: *Trypanosoma brucei*, Lab 110 EATRO (veterinary parasite); *Trypanosoma rhodesiense* KETRI 243 (human isolate), *T. rhodesiense* 243As 10-3 (clone of KETRI 243 highly resistant to melarsoprol and pentamidine). Usually 2 -3 growth curves are necessary to determine an IC_{50} value. Each experiment is incubated at 37°C for 72 h in 5% CO_2.

b) Trichomonads. The method used was the minimal inhibitory concentration (MIC) assay developed by Meingassner et al. (1978). Strains used were *T. vaginalis* C1-NIH (ATTC 30001) and a metronidazole-resistant strain, CDC-085 (ATCC 50143) and KV-1, a *Tritrichomonas foetus* extract. These are incubated aerobically in 96 well plates with triplicate serial dilutions of each extract, and counted microscopically at 24 and 48 h.

c) *In vivo* studies. For African trypanosomes, extracts having IC_{50} values of $\leq$ 20 µg/ml were tested in a *T. brucei* Lab 110 EATRO mouse model infection (Bacchi et al., 1990). Mice (10–25 g) were infected with 5×10^5 trypanosomes and treatment was begun 24 h later. Since all extracts were solubilized in 50% DMSO, extracts were diluted in this solvent to achieve correct concentrations. Mice (3 per dose point) were dosed once daily for three days, by the intraperitoneal route. Animals surviving > 30 days with no evidence of parasites in blood smears are considered cured.

2. Results

a) African trypanosomes. A total of 85 extracts were received from Dr. Chris Okunji of WRAIR (12 were a resupply) and 34 from Drs. Apollinaire Tsopmo and Pierre Tane (University of Dschang, Cameroon, AP#2) during the reporting period. In trypanosome growth screens, 31 were tested vs. 3 isolates and initial tests (*T. brucei* only) were done on another 25 extracts (Table 1). Two of the WRAIR extracts (#2202 and #2008) were not soluble in DMSO or water and were not tested. A total of 24 of the 66 WRAIR extracts and 5 of the 8 AP#2 extracts had IC_{50} values $\leq$ 20 µg/ml (Table 1 and 2). Of the WRAIR extracts, 6 of the 24 most active had IC_{50} values $\leq$ 1 µg/ml (Table 1). Of the AP#2 extracts, 4 had IC_{50} values of $\leq$ 5 µg /ml and 1 had

IC_{50} values of ~ 1 μg/ml (Table 2). Some of the latter data, concerning extracts from *Aframomum* has recently been published (Kamnaing et. al., 2003).

The 18 WRAIR extracts having most activity (IC_{50} values ≤ 5 μg/ml and ≤ 1 μg/ml) are listed in Table 3. Sixteen of these (excepting 2192 and 2200) were tested in the *T. brucei in vitro* screen (Bacchi et al., 1997). Mice (25 – 30 g) were infected with 5 x 10^5 trypanosomes and the infection allowed to develop for 24 h. Plant extracts were diluted to 5 mg/ml with 50% DMSO. Mice (groups of 3) were injected (i.p.) with a dose range of 1, 5, 10 and 25 mg/kg extract once daily for 3 days. Control mice died in 4 – 5 days. The life-span of treated mice was compared to untreated infected controls. None of the extracts were curative at the above doses, and none were obviously toxic. We have some of the most active extracts left and intend to attempt twice a day (B.I.D) dosing at 25 and 50 mg/kg.

b) Trichomonads. A total of 8 extracts were tested against metronidazole-sensitive (ATCC 30001) and –resistant (ATCC 50143) isolates (Table 4). Two extracts (#1863 and #1868) had MIC values ≤ 0.6 mg/ml for the sensitive isolate and 4 had values of ≤ 0.6 mg/ml for the resistant isolate. The active extracts were from *Aspilia, Combretum, Enantia, Hoslundia, Mormadica, Phyllanthus,* and *Cleistopholis*. The *T. vaginalis* strains have presented problems due to contamination and failure to grow well in normal medium, and efforts are continuing to reassess the growth medium and resume normal progress.

A total of 31 extracts were tested vs. *Trichomonas foetus* (Table 5). Of these, 4 had MIC values of 0.6 mg/ml. These were from *Hoslundia* (#1874), *Phyllanthus* (#1879), *Cleistopholis* (#1985, CH_2Cl_2; #1896, MeOH).

Of the extracts tested vs. trypanosomes and trichomonads, and found to be highly growth inhibitory (Table 4 and 7), only 4 were active vs. both types of organisms, indicating that most extracts had some degree of specificity and that growth inhibitory activity would not be a function of general toxicity.

c) Training. Part of the function of Haskins Laboratories at Pace University is to train undergraduates in research techniques. In this reporting period, a total of 4 undergraduates participated in this research, and will present papers on their work at the Dyson College Society of Fellows Workshop (Arts and Sciences Honor Society). Many other undergraduates have similarly taken part in this work over the past 10 years. In addition, the Woodrow Wilson Foundation, joint with NSF, annually holds a two week High School Science Teacher Summer Training Institute at Pace University. This program features the ecosystem as a major topic and the students are given lectures and laboratory demonstrations concerning biodiversity, conservation of ecosystem resources and the activities of the ICBG program.

Issues of concern are resolving culture problems with *T. vaginalis* extracts, and the identification of active agents in those plant extracts which are highly growth inhibitory vs. trichomonads and trypanosomes.

III. Key Research Accomplishments

- Identification of 5 new extracts having IC_{50} values of $\leq$ 1 μg/ml in the trypanosome screen. These were from *Melian, Culcasis, Holorrhena, Jatropha,* and *Boerrhavia.*
- The methanol extract of *Holarrhena floribunda* leaves was far more active than the CH_2 Cl_2 extract in the trypanosome screen, indicating selective extraction of an active agent.
- The most active extracts vs. trichomonads were not those most active vs. African trypanosomes indicating some degree of specificity.
- Identification of 7 extracts active at MIC values of $\leq$ 0.6 mg/ml vs. trichomonads.
- Since the most active extracts are crude material, we are hopeful that the active agents can be identified through subfractionation.

IV. Reportable outcomes

Kammnaing, P., Tsopmo, P., Tanifum, E.A., Tane, P., Ayafor, J.F., Sterner, O., Rattendi, D., Iwu, M.M., Schuster, C., and Bacchi, C.J. 2003. Diarylheptanoids from *Afromomum letestuianam* K. Schum (Zingiberaceae). J. Natural Products, 66:364-367.

Tsopmo, P., Kammnaing, P., Ngamga, D., Tane, P., Ayafor, J.F., Sterner, O., Rattendi, D., Iwu, M.M., Schuster, C., and Bacchi, C.J. Antitrypanosomal alkaloids from *Xymalos monospora.* J. Natural Products (under review).

V. Conclusions

We intend to further assess more refined extracts of primary extracts found highly growth inhibitory in the past reporting period and earlier periods, including extracts or purified compounds from a group of compounds termed cryptolepins which are found in several genera under study, and which may have antiprotozoal activity. Table 4 lists the most active of the recent extracts. Most of these gave IC_{50} values $\leq$ 20 μg/ml. Some were active at $\leq$5 μg/ml, but a few had IC_{50} values below 1 μg/ml. The latter include: *Melian excelsa* (stem bark), *Holarrhena floribunda* (Leaves), *Jatropha curcas* (leaves), *Boerrhavia diffusa* (roots). Additional subfractions of these plant extracts have been recently supplied and are scheduled for testing. Based on this work and earlier studies (prior to Jan.2002), we have received supplies of the following extracts for further *in vitro* and *in vivo* testing: SU2158 *Hyptis suaveolens*, SU2157 *Culcasia scandens*, SU1875 *Icacina trichanta*, SU1891 *Uvaria chamae* (for trypanosomes); also SU1870 *Combretum dulchipetalum*, SU1874 *Hoslundia opposita*, SU1876 *Cleistopholis patens* and SU2152 *Solenostemon monostachyus* (for trichomonads).

The most recent data from the trichomonad assays are listed in Tables 4, 5, and 6. These have lagged behind the trypanosome assays because of difficulties in culture of stabilates (particularly *T. vaginalis* NIH-1) and some contamination problems. Nevertheless, a number of extracts had significant activity (MI6 $\leq$ 0.6 mg/ml (Table 7) and will be tested *in vivo* in the *T. vaginalis* subcutaneous mouse assay (Honigberg et.al. 1966).

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Table 1. IC_{50} values for plant extracts received from WRAIR (Jan. 2002 – Jan. 2003). Compounds were tested vs. trypanosome isolates grown in blood forms in HMI-18 medium containing 20% fetal bovine serum. Coulter counts were made daily and IC_{50} values determined after 48 h as described in the text. (Data thru April 2003).

	IC_{50} (µg/ml)		
	Lab110 EATRO	KETRI 243	KETRI 243 As10-3
SU-2140	2.8	10	8.7
SU-2141	44% @ 500 µg/ml	+ @ 500 µg/ml	-
SU-2142	25.5	21	55
SU-2143	330	500	11% @ 500 µg/ml
SU-2144	67	50	43
SU-2145	6.6	5.5	
SU-2146	28.5	16.5	34
SU-2147	250	500	96
SU-2148	4.0	1.575	6.2
SU-2149	12	19.25	22.5
SU-2150	55	112.5	125
SU-2151	0.525	0.525	1.25
SU-2152	71	32	22.0
SU-2153	27.5	22.5	88
SU-2154	66	71	100
SU-2155	9.3	22.5	64
SU-2156	5.8	77	12.5
SU-2157	0.235	19.5	3.85
SU-2158	0.1	3.7	2.45
SU-2159	7.5	12.25	14.0
SU-2160	25% @ 500 µg/ml	44% @ 500 µg/ml	-
SU-2161	43% @ 500 µg/ml	44% @ 500 µg/ml	-
SU-2162	4.6	61	51.5
SU-2163	5.0	10	22.0
SU-2164	0.5	6.7	6.8
SU-2165	3.8	16.5	27.0
SU-2166	5.0	20.0	18.75
SU-2167	0.195	16.5	17.0
SU-2168	20% @ 500 µg/ml	41% @ 250 µg/ml	157.5
SU-2169	11% @ 500 µg/ml	80	71.0
SU-2170	17% @ 500 µg/ml	500	31% @ 500 µg/ml
SU-2171	42.5	55.5	
SU-2172	145	75.6	
SU-2175	50		
SU-2176	23.5		
SU-2177	19.0		
SU-2180	9.5		
SU-2181	59		
SU-2192	2.2		

Table 1 (continued)

	IC_{50} (μg/ml)		
	Lab110 EATRO	KETRI 243	KETRI 243 As10-3
SU-2194	16.5		
SU-2195	71		
SU-2196	120		
SU-2197	18.5		
SU-2198	24.0		
SU-2200	0.92		
SU-2201	77		
SU-2202			
SU-2203	37		
SU-2204	70		
SU-2205	135		
SU-2206	6.75		
SU-2207	28.75		
SU-2208			
SU-2209	16		
SU-2210	25		
SU-2211	3.5		
SU-2212	63.5		
SU-2213	35% @ 500 μg/ml		
Melarsoprol	0.0075	0.016	0.016
Pentamidine	0.0008	0.00098	0.00075

Table 2. Activity of University of Dschang (AP #2) extracts vs. African trypanosomes *in vitro*. Data to March 2003.

	IC_{50} (μg/ml)		
		KETRI	
	Lab110 EATRO	**243**	**243 As 10-3**
ASP	0.5	1.5	3.9
ASS_2	28	26.5	130
ASS_4	30.5	16	51
ASS_5	55	50.1	56
TZM_{1A}	21.5	18	15.9
TZM_1	2.35	22.5	17
TZM_4	4.45	2.1	1.85
TZM_4 HCl	3.59	3.59	1.80
TZM_5	25	-	-
NMG-2	125	-	-
NMG-2	8.65	-	-
NMG-5	16.0	-	-
NMG-6	0 @500 μM	-	-

Table 3. WRAIR extracts, received through April 2003 having significant activity $IC_{50} \geq 20$ µg/ml vs. African trypanosomes *in vitro: T. b. brucei* Lab 110 EATRO.

Extract	Plant	Extract	Plant
SU 2140*	*Premma guadrifolia*	SU 2162*	*Cassytha filiformis* (whole plant)
SU 2145	*Cassia siamea* (leaves/stems) (dry)	SU 2163	*Cassytha filiformis* (whole plant)
SU 2148*	*Guarea thompsonli* (Stem bark)	SU 2164**	*Holarrhena floribunda* (leaves)
SU 2149	*Guarea thompsonli* (Stem bark)	SU 2165*	*Holarrhena floribunda* (leaves)
SU 2151**	*Melian excelsa* (Stem bark)	SU 2166	*Jatropha curcas* (leaves)
SU 2156	*Goyania long pelara* (Leaves/stems)	SU 2167**	*Jatropha curcas* (leaves)
SU 2157**	*Culcasis scanders* (whole plant)	SU 2180	*Combretum dulchipetalum* (leaves)
SU 2158*	*Hyptis suaveolens* (leaves)	SU2192*	*Renealmia porypus*
SU 2159	*Hyptis suaveolens* (leaves)	SU 2200**	*Boerrhavia diffusa* (roots)

*Very active, $IC_{50} < 5$ µg/ml; **Most active, $IC_{50} \leq 1$ µg/ml.

Table 4. Activity of WRAIR extracts *in vitro* vs. *Trichomonas vaginalis* strains. ATCC 30001 is metronidazole sensitive and ATCC 50143 is resistant. MIC, Minimal Inhibitory Concentration.

	MIC (mg/ml)			
	ATCC 30001		ATCC 50143	
Extract	**24h**	**48h**	**24h**	**48h**
SU 1863	1.3	0.6	<1.3	0.6
SU 1864	1.3	<1.3	<1.3	<1.3
SU 1865	<1.3	<1.3	<1.3	<1.3
SU 1868	1.3	0.6	1.3	1.3
SU 1870	-	-	0.3	0.3
SU 1873	-	-	1.3	0.6
SU 1876	-	-	<1.3	<1.3
SU 1877	-	-	<1.3	0.6
Metronidazole	-	0.003	-	0.40

Table 5. Activity of WRAIR extracts *in vitro* vs. *T. foetus* KV1

	MIC (mg/ml)			MIC (mg/ml)	
Extract	24 h	48 h	Extract	24 h	48 h
SU 1863	1.3	–	SU 1884	1.3	1.3
SU 1864	>1.3	–	SU 1887	>1.3	–
SU 1865	>1.3	–	SU 1889	1.3	–
SU 1866	1.3	1.3	SU 1890	>1.3	–
SU 1867	>1.3	>1.3	SU 1891	>1.3	1.3
SU 1869	>1.3	1.3	SU 1892	>1.3	>1.3
SU 1872	>1.3	1.3	SU 1893	1.3	1.3
SU 1873	1.3	–	SU 1894	>1.3	>1.3
SU 1874	0.6	0.3	SU 1895	0.63	0.63
SU 1875	>1.3	1.3	SU 1896	0.31	–
SU 1878	1.3	1.3	SU 1898	>1.3	–
SU 1879	1.3	0.6	SU 1899	1.3	1.3
SU 1880	>1.3	1.3	SU 1900	>1.3	>1.3
SU 1881	>1.3	1.3	SU 1901	>1.3	>1.3
SU 1882	1.3	1.3	SU 1902	1.3	1.3
SU 1883	>1.3	1.3	Metronidazole	–	0.003

Table 6. Most active WRAIR extracts vs. Trichomonads (MIC $\leq$ 0.6 mg/ml)

Lab Number	Plant Name	Extract
l*SU 1863	*Aspilia africana*	CH_2Cl_2
SU 1868	*Combretum dulchipetalim*	MeOH
*SU 1870	*Combretum dulchipetalim*	Aq.
*SU 1873	*Enantia chlorontha*	MeOH
*SU 1874	*Hoslundia opposita*	CH_2Cl_2
SU 1877	*Mormodica charanta*	CH_2Cl_2
SU 1879	*Phyllanthus amerus*	Aq.
SU 1895	*Cleistopholis patent*	CH_2Cl_2

* Also active vs. trypanosomes

Trypanocidal Diarylheptanoids from *Aframomum letestuianum*

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Received August 23, 2002

Three new diarylheptanoids, (4*Z*,6*E*)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-4,6-dien-3-one, letestuianin A (**1**), (4*Z*,6*E*)-5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-4,6-dien-3-one, letestuianin B (**2**), and 1,7-bis(4-hydroxyphenyl)heptan-3,5-dione, letestuianin C (**3**), as well as the known (4*Z*,6*E*)-5-hydroxy-1,7-bis(4-hydroxyphenyl)hepta-4,6-dien-3-one (**5**) were isolated from *Aframomum letestuianum*. The known flavonoids 3-acetoxy-5,7,4′-trihydroxyflavanone, 3-acetoxy-7-methoxy-5,4′-dihydroxyflavanone, 7-methoxy-3,5,4′-trihydroxyflavone, and 3,3′,4′,5,7-pentahydroxyflavan were also obtained from this plant. Their structures were determined using a combination of 1D and 2D NMR techniques. The four diarylheptanoids were tested for growth inhibitory activity in vitro versus bloodstream forms of African trypanosomes. IC_{50} values in the range of 1−3 μg/mL were found for compounds **3** and **5**.

The genus *Aframomum* K. Schum belongs to the economically and medicinally important family Zingiberaceae. It is represented in Cameroon by over 20 species of rhizomatous herbs.[1] All of them are widely used locally in ethnodietary and in folk medicinal preparations as well as for cultural and spiritual purposes.[2] In our previous research on this genus, we reported the isolation and characterization of several flavonoids and labdane diterpenes.[3−5] In continuation of our work on this genus and as part of our efforts to discover new antiparasitic drug leads from Cameroonian medicinal plants[6] we have investigated the seeds of *Aframomum letestuianum* and herein report the isolation of four diarylheptanoids. Three are new compounds to which we have given the trivial names letestuianin A (**1**), letestuianin B (**2**), and letestuianin C (**3**). The fourth is the previously reported (4*Z*,6*E*)-5-hydroxy-1,7-bis(4-hydroxyphenyl)hepta-4,6-dien-3-one (**5**).[7] In addition, the known flavonoids 3-acetoxy-5,7,4′-trihydroxyflavanone,[4] 3-acetoxy-7-methoxy-5,4′-dihydroxyflavanone,[4] 7-methoxy-3,5,4′-trihydroxyflavone,[5] and 3,3′,4′,5,7-pentahydroxyflavan[8] were isolated in large quantitities. The trypanocidal activity of the diarylheptanoids is presented.

Results and Discussion

A sample of the air-dried powdered seeds of *A. lestestulanum* was extracted with MeOH−CH_2Cl_2 and subjected to sequential extraction with hexane and CH_2Cl_2. Bioassay-guided fractionation and purification of the CH_2Cl_2-soluble fractions led to the isolation of four diarylheptanoids and four flavonoids. The structures of the compounds were elucidated by spectroscopic techniques, and comparison with literature data revealed that three of the isolated diarylheptanoids are new compounds.

Compound **1** was obtained as a yellowish oil. The EIMS spectrum showed a molecular ion peak at *m/z* 340 with 100% intensity, compatible with the molecular formula $C_{20}H_{20}O_5$. The IR spectrum showed important absorption bands at ν_{max} 3363 (OH) and 1633 cm^{-1} (C=C−C=O). The ^1H NMR spectrum revealed the presence of a *para*-disubstituted benzene ring characterized by signals at δ 7.52 (2H, d, J = 8.5 Hz) and 6.88 (2H, d, J = 8.5 Hz); a 1,3,4-trisubstituted benzene ring [δ 6.85 (H-2″, d, J = 2.0 Hz), 6.72 (H-5″, d, J = 8.4 Hz), and 6.68 (H-6″, dd, J = 8.4, 2.0 Hz)]; a pair of *trans* olefinic protons at δ 7.53 (H-7, d, J = 15.9 Hz) and 6.53 (H-6, d, J = 15.9 Hz); a methoxy signal at δ 3.80 (s); and two methylenes at δ 2.85 and 2.67 (each triplet, J = 8.1 Hz). This was in sound agreement with the ^{13}C NMR spectrum (Table 1), which showed signals attributed to a carbonyl at δ 199.9 (C-3) and a hydroxylated olefinic carbon at δ 178.5, which with subsequent HMBC cross correlation peak with the *trans* olefinic protons as well as with H-4 (δ 5.81) was attributed to C-5. Three oxygenated sp^2 carbon atoms were also observed at δ 145.9, 148.3, and 160.5. A judicious analysis of the ^1H−^1H COSY data of **1** implied connectivities of H-7 to H-6, H-2 to H-1, H-2′ to H-3′ and H-6′, H-5′ to H-3′ and H-6′, and H-6″ to H-2″ and H-5″. The correlations observed in the NOESY and HMBC spectra attached the methoxy group at position C-3″ rather than C-4″, and pertinent correlation peaks were observed between the OMe group (δ 3.80) and H-2″ (δ 6.85) in the NOESY spectrum and between the OMe protons and C-3″ in the HMBC spectrum. The stereochemistry of the C-6/C-7 double bond bond is *E* as judged by the coupling constant between the two protons (J = 15.9 Hz), and that of the C-4 double bond is *Z*, as a clear NOESY correlation peak was observed between H-4 and H-6. Further analysis of HMBC and NOESY spectra led to the assignment of all carbons and protons, and the structure of compound **1** is (4*Z*,6*E*)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-4,6-dien-3-one. The trivial name letestuianin A was given to this new diarylheptanoid.

Compound **2** was obtained as yellow needles (CH_2Cl_2), mp 179−180 °C. The EIMS of **2** showed a molecular ion peak at *m/z* 370 compatible with the molecular formula $C_{21}H_{22}O_6$. The IR spectrum showed absorption bands due

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10.1021/np020362f CCC: $25.00
Published on Web 02/19/2003

Table 1. ^{13}C (125 MHz) and ^{1}H NMR (500 MHz) Data for Compounds **1**, **2**, and **3**

	1[a]		**2**[b]		**3**[c] (major component)	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	31.7	2.85 t (8.1)	30.4	2.77 t (8.0)	29.7	2.71 s
2	42.7	2.67 t (8.1)	41.3	2.65 t (8.0)	46.4	2.71 s
3	199.5		199.2		206.5	
4	101.0	5.81 s	100.2	5.90 s	57.4	3.52 s
5	178.5		177.9		206.5	
6	120.8	6.53 d (15.9)	119.7	6.63 d (15.9)	46.4	2.71 s
7	140.6	7.53 d (15.9)	140.2	7.45 d (15.9)	29.7	2.71 s
1′	127.8		126.3		133.0	
2′	130.9	7.52 d (8.5)	111.0	7.25 d (2.0)	130.4	6.98 d (8.5)
3′	116.9	6.88 d (8.5)	148.0		116.3	6.68 d (8.5)
4′	160.5		149.2		156.7	
5′	116.9	6.88 d (8.5)	115.7	6.78 d (8.0)	116.3	6.68 d (8.5)
6′	130.9	7.52 d (8.5)	123.0	7.13 dd (8.0, 2.0)	130.4	6.98 d (8.5)
1″	133.4		131.6		133.0	
2″	112.9	6.85 d (2.0)	112.5	6.77 d (2.0)	130.4	6.98 d (8.5)
3″	148.3		147.4		116.3	6.68 d (8.5)
4″	145.9		144.7		156.7	
5″	115.8	6.72 d (8.4)	115.3	6.64 d (7.9)	116.3	6.68 d (8.5)
6″	121.6	6.68 dd (8.4, 2.0)	120.3	6.59 dd (7.9, 2.0)	130.4	6.98 d (8.5)
OMe′			55.7	3.78 s		
OMe″	56.3	3.80 s	55.5	3.71 s		

[a] Spectra recorded in acetone-d_6. [b] Spectra recorded in DMSO-d_6. [c] Spectra recorded in CD_3OD.

to hydroxyl group(s), enone, and aromatic ring(s) functionalities at ν_{max} 3436, 1631, and 1602 cm^{-1}, respectively. The ^{1}H and ^{13}C NMR data (Table 1) of **2** were closely related to those of compound **1**. The only significant differences compared to **1** are that both aromatic systems are 1,3,4-trisubstituted and the presence of an additional methoxy group in **2**. Once more the NOESY spectrum was useful for the determination of the position of the methoxy groups on the aromatic rings as well as for the *Z* conformation of one of the double bond. Important correlation peaks were observed between the OMe at δ 3.71 and the proton at δ 6.77 (d, $J = 2.0$ Hz) as well as the OMe at δ 3.78 and the proton at δ 7.25 (d, $J = 2.0$ Hz). Together with COSY and HMBC data the structure (4*Z*,6*E*)-5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-4,6-dien-3-one was determined for compound **2**, and it was given the trivial name letestuianin B.

Compound **3** was obtained as a pale yellow oil. The EIMS spectrum of **3** showed a molecular ion peak at *m/z* 312 compatible with the molecular formula $C_{19}H_{20}O_4$. The IR spectrum showed absorption bands at ν_{max} 3407, 1630, 1613, 1515, and 828 cm^{-1} closely related to those of **1** and **2**. The 1D NMR spectra suggested the presence of two components, in a 3:7 ratio. For the major component, the ^{1}H NMR spectrum indicated the presence of a *para*-disubstituted benzene ring [δ 6.98 (2H, d, $J = 8.5$ Hz) and 6.68 (2H, d, $J = 8.5$ Hz)] and two methylenes appearing as singlet at δ 2.71. An isolated proton appeared at δ 3.52 as a singlet. The intensity of the latter signal was very low due to exchange with deuterium from the methanol solvent used for NMR experiments. These data account only for nine protons, and the fact that only eight carbon signals appeared in its ^{13}C NMR spectrum suggests that **3** is symmetric with two identical benzene rings. The data for the major component were compatible only with the 1,3-diketone shown in Figure 1, and as expected, this is in equilibrium with an enol tautomer. Typical signals for the enol appeared in the ^{1}H and ^{13}C NMR spectra, for example a proton signal at δ 4.58 (H-4) and carbon signals at δ 194.7 (C-3) and 100.0 (C-4), but to confirm this tautomeric equilibrium, compound **3** was treated with a mixture of pyridine–Ac_2O (1:1) to give the acetylated derivative **4**. The analysis of the ^{1}H NMR spectrum of **4** revealed the presence of a 1,4-disubstituted benzene ring, showing that

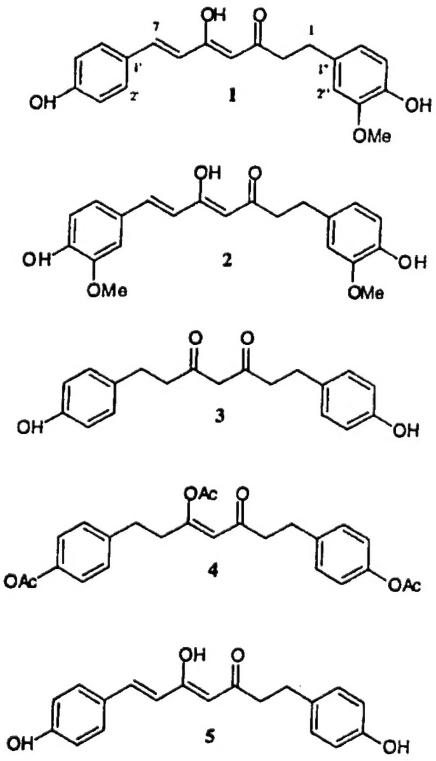

Figure 1.

the symmetric nature of the molecule had been distorted. An olefinic signal was also observed in **4** at δ 5.42 in replacement of the methylene signal that was present at δ 3.52 in **3**. The presence of three acetate functions was characterized by shifts at δ 2.22 (6H, s) and 2.10 (3H, s). The analysis of the ^{13}C NMR spectrum of **4** with signals at δ 169.5, 169.7, and 170.0 confirmed the three acetate functions. A conjugated carbonyl function was also observed at δ 193.2. All the above information showed that **4** was the enol form of **3**. Further analysis of HMBC, COSY, and NOESY spectra of the nonacetylated and acetylated derivative led to the characterization of compound **3** as 1,7-bis(4-hydroxyphenyl)heptan-3,5-dione, consequently named letestuianin C.

Table 2. Antitrypanocidal Activities of *Aframomum letestuianum* Diarylheptanoids

	IC_{50} (μg/mL)		
	Lab110 EATRO	KETRI *T. b. rhodesiense* KETRI isolates	
compound	*T. b. brucei*	243	243 As 10−3
1	>100		
2	67	>100	>100
3	1.4	2.3	2.6
5	2.6	2.8	1.3
melarsoprol	0.002	0.0005	0.005
pentamidine	0.0006	0.0005	0.004

Previous studies on the genus *Aframomum* have, up to date, reported the presence of only two major classes of natural products, diterpenoids and flavonoids. To the best of our knowledge, **1**, **2**, **3**, and **5** are the first diarylheptanoids reported from this important genus, although they are common in the sister genera *Alpinia*[9-11] and *Curcuma*.[12-14] The four diarylheptanoids obtained were assayed for trypanocidal activity, tested against bloodstream forms of *Trypanosoma b. brucei* and *Trypanosoma b. rhodesiense* isolates grown in vitro in 24-well plates. Coulter counts were made daily, and the IC_{50} values determined after 48 h are given in Table 2. Compound **1** was not growth inhibitory below 100 μg/mL. Compound **2** gave an IC_{50} value of 67 μg/mL with the *T. b. brucei* isolate but >100 μg/mL with *T. b. rhodesiense* isolates. Compounds **3** and **5**, however, were highly effective in the range 1−3 μg/mL for all isolates tested. Interestingly, the additional methoxy group in **1**, compared to **5**, makes it inactive. Corresponding IC_{50} values for the trypanocides melarsoprol and pentamidine were ~300−5000-fold lower; however, lack of sufficient material prevented us from testing these compounds in vivo in a mouse model infection.

Experimental Section

General Experimental Procedures. Melting points were recorded with a Reichter microscope and are uncorrected. The UV and IR spectra (KBr) were recorded with a Shimadzu UV-3001 and a Jasco FT-IR spectrophotometer, respectively. ^1H NMR and ^{13}C NMR were recorded in $CDCl_3$, acetone-d_6, DMSO-d_6, or CD_3OD using a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The chemical shifts (δ) are reported in parts per million relative to tetramethylsilane (TMS, $\delta = 0$), while the coupling constants (J) are given in Hz. COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH} = 145$ Hz and $^nJ_{CH} = 10$ Hz. The raw data were transformed and the spectra evaluated with the standard Bruker UXNMR software. The positive EI (70 eV) and CI mass spectra were recorded with a JEOL SX102 spectrometer. Column chromatography was run on Merck Si gel 60 and gel permeation on Sephadex LH-20. TLC analyses were carried out on Si gel GF_{254} precoated plates with detection accomplished by spraying with 50% H_2SO_4 followed by heating at 100 °C, or by visualizing with a UV lamp at 254 and 366 nm.

Plant Material. The seeds of *A. letestuianum* were collected from Abong-bang, East Province, Cameroon, in December 1998. Mr. Paul Mezili, a retired botanist of the Cameroon Herbarium, authenticated the plant material. Voucher specimens (BUD 0391) were deposited at the Herbarium of the Botany Department of the University of Dschang.

Extraction and Isolation. The air-dried powdered seeds of *A. letestuianum* (2 kg) were macerated with a mixture (1:1) of MeOH−CH_2Cl_2 (4 L) overnight and evaporated in vacuo to yield a crude extract (150.5 g). This crude extract was dissolved in 80% MeOH (600 mL) and extracted hexane (3 × 500 mL). The aqueous MeOH was further diluted with water to 60%MeOH and extracted with CH_2Cl_2 (3 × 500 mL). Vacuum concentration yielded CH_2Cl_2 extract (36.5 g) and hexane extract (28.0 g), which contained mostly fats. Subjection of the CH_2Cl_2 extract to column chromatography over silica gel eluting with a CH_2Cl_2−hexane gradient followed by acetone−CH_2Cl_2 afforded three major fractions, I [500 mg, CH_2Cl_2−hexane (6:4)], II [16.0 g, CH_2Cl_2−hexane (8:2) and acetone−CH_2Cl_2 (1:9)], and III [2.1 g, acetone−CH_2CL_2 (2:8)]. Subjecting fraction I to repeated column chromatography on silica gel eluted with a CH_2Cl_2−hexane gradient and further purification by gel permeation chromatography on Sephadex LH-20 (MeOH) afforded compounds **1** (24 mg), **2** (10.4 mg), and 7-methoxy-3,5,4′-trihydroxyflavanone (5.5 mg). Subjection of fraction II (7.5 g) to gel permeation chromatography on Sephadex LH-20 (MeOH) gave additional amount of **1** (15 mg), 3-acetoxy-5,7,4′-trihydroxyflavanone (3.5 g), 3-acetoxy-7-methoxy-5,4′-dihydroxyflavanone (1.8 g), and a mixture of two main products (350 mg), which was further purified by countercurrent chromatography (CCC) eluting head to tail with hexane−ethyl acetate−MeOH−H_2O (4:6:5:5) and reversing the flow after 3 h to obtain compounds **3** (179 mg) and **5** (86 mg). Treatment of fraction III on a silica gel column eluted with MeOH−CH_2Cl_2 gradient followed by gel permeation on Sephadex LH-20 (MeOH−CH_2Cl_2, 1:1) afforded 3,3′,4′,5,7-pentahydroxyflavan (139 mg) and a mixture of nonresolved compounds.

(4*Z*,6*E*)-5-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-4,6-dien-3-one, letestuianin A (1): yellowish oil; UV (MeOH) λ_{max} (log ε) 380 (3.2) and 283 (3.9) nm; IR (KBr) ν_{max} 3363, 2937, 1633, 1583, 1514, 1431, 831, and 790 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 340 $[M]^+$ (100), 322 (10), 189 (30), 147 (70), 137 (55), 107 (18); HREIMS *m/z* 340.1304 (calcd for $C_{20}H_{20}O_5$, 340.1311).

(4*Z*,6*E*)-5-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-hepta-4,6-dien-3-one, letestuianin B (2): shiny yellow needles (CH_2Cl_2−hexane); mp 179−180 °C; UV (MeOH) λ_{max} (log ε) 374 (2.9) and 288 (3.4) nm; IR (KBr) ν_{max} 3436, 1631, 1602, 1511, 1280, 1202, 1028, and 814 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 370 $[M]^+$ (44), 352 (16), 219 (18), 177 (63), 137 (100), 44 (25); HREIMS *m/z* 370.1411 (calcd for $C_{21}H_{22}O_6$, 370.1416).

1,7-Bis(4-hydroxyphenyl)heptan-3,5-dione, letestuianin C (3): yellowish oil; UV (MeOH) λ_{max} (log ε) 279 (3.4) and 224 (2.4) nm; IR (KBr) ν_{max} 3407, 1623, 1613, 1515, 1462, 1385, 1243, 828 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 312 $[M]^+$ (34), 191 (10), 120 (20), 107 (100), 77 (10); HREIMS *m/z* 312.1358 (calcd for $C_{19}H_{20}O_4$, 312.1361).

Acetylation of Letestuianin C (3). Compound **3** (25 mg) was dissolved in a (1:1) mixture of pyridine−Ac_2O (4 mL) and the reaction mixture left at room temperature overnight. The product was concentrated with addition of toluene and purified on a silica gel column (hexane−EtOAc, 9:1) to give 5-acetoxy-1,7-bis(4-acetoxyphenyl)hepta-4-en-3-one (**4**) (26 mg) as a colorless oil: ^1H NMR ($CDCl_3$, 500 MHz) δ 2.10 (Ac), 2.22 (2 × Ac), 2.52 (4H, t, $J = 7.6$ Hz, H-2, H-6), 2.81 (4H, t, $J = 7.6$ Hz, H-1, H-7), 5.42 (H-4, s), 6.90 (4H, m, H-2′, H-6′, H-2″, H-6″), 7.23 (4H, m, H-3′, H-5′, H-3″, H-5″); ^{13}C NMR $CDCl_3$, 125 MHz) δ 31.2 (C-1, C-7), 40.3 (C-2, C-6), 100.1 (C-4), 121.9 (C-3″, C-5″), 122.0 (C-3′, C-5′) 129.7 (C-2″, C-6″), 129.8 (C-2′, C-6′), 138.5 (C-1″), 138.6 (C-1′), 149.4 (C-4′, C-4″), 179.1 (C-5), 193.2 (C-3).

Biological Assay. Assays for inhibition of trypanosomal growth were conducted as previously described.[15,16] Bloodstream-form trypanosomes were cultured in modified IMDM with 20% horse serum at 37 °C. Drug studies were done in duplicate in 24-well plates (1 mL/well) with final inhibitor concentrations of 0.1, 1, 10, 25, and 100 μg/mL. Wells were inoculated with 10^5 trypanosomes, and one-half the volume of each well was changed daily. After 48 h, the parasite number was determined in a Model Z1 Coulter counter and IC_{50} values were calculated from semi-log plots. Assays were done two or more times, using widely spaced concentration curves initially, followed by curves of closely spaced values to obtain the IC_{50} value.

Compounds were dissolved in 100% dimethyl sulfoxide and diluted in medium, so that the dimethyl sulfoxide concentration never exceeded 0.3%, a noninhibitory concentration.

Strains used were *Trypanosoma b. brucei* Lab 110 EATRO and Kenya Trypanosomiasis Research Institute (KETRI) isolates *Trypanosoma b. rhodesiense* 243 and 243 As 10-3.[15,16]

Acknowledgment. The authors gratefully acknowledge financial support from the Fogarty International Center (NIH Grant 1U01TWO 0317, as part of the International Cooperative Biodiversity Group Program) and the Swedish Natural Science Research Council.

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NP020362F